

Host Cell DNA Chain Initiation Protein Requirements for Replication of Bacteriophage G4 Replicative-Form DNA

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Bacteriophages G4ev1 and G4bs1 are simple temperature-resistant derivatives of wild-type G4 as demonstrated by restriction endonuclease analyses. The rate of replication of the duplex replicative-form DNA of these phages was normal in *dnaB* and *dnaC* mutants of the host, whereas the rate was markedly reduced in a *dnaG* host mutant at the restrictive temperature. We conclude that G4 duplex DNA replication requires the host cell *dnaG* protein, but not the *dnaB* and *dnaC* proteins. The reasons for the differences between our conclusions and those based on previously published data are documented and discussed.

The *Escherichia coli dnaG* protein catalyzes the synthesis of an oligonucleotide primer on bacteriophage G4 DNA in vitro without the assistance of the *dnaB* and *dnaC* initiation proteins (1, 14, 17). This also seems to be true in the cell, where the infecting single-stranded G4 DNA molecule serves as template for the synthesis of the complementary strand (11).

In vivo studies of the host cell initiation protein requirements for replication on the duplex G4 replicative-form (RF) DNA are complicated by the inherent temperature sensitivity of G4 RF DNA replication. It is desirable to use a temperature-resistant strain of G4 when studying the effects of temperature-sensitive host DNA synthesis mutations on G4 RF DNA replication. To this end, the host cell requirements for duplex RF DNA replication of a temperature-resistant bacteriophage, designated G4tr1, have been shown to include *dnaB* and *dnaC* proteins, as well as the *dnaG* protein (3). These experiments, together with those of Kodaira and Taketo using wild-type G4 (12), have been interpreted as demonstrating that bacteriophage G4 requires the host cell *dnaB* and *dnaC* proteins, together with the *dnaG* protein, for the replication of its duplex RF DNA.

Recent experiments in our laboratory led us to question the assignment of bacteriophage G4tr1 as a simple variant of wild-type G4. Therefore, we compared the structures of the RF DNA molecules isolated from cells infected with G4tr1, wild-type G4, and two other temperature-resistant strains of G4. We found G4tr1 to be different from G4. Thus, G4tr1 is not an appropriate probe for determining the requirements for G4 RF DNA replication.

We attempted to use wild-type G4 to determine the requirements for host cell DNA chain

initiation proteins. However, the inherent temperature sensitivity of wild-type G4 made the interpretation of several key experiments extremely difficult. In view of this, we decided that wild-type G4 also should not be used to probe this event. This prompted us to reexamine the replication of G4 duplex RF DNA in host cell DNA synthesis-defective mutants, using temperature-resistant strains of G4.

MATERIALS AND METHODS

Bacteria and phage strains. *E. coli* strains LD311 [*uvrA*⁻ *thyA*⁻ *endI*⁻ *dnaB*(Ts)] and LD332 [*uvrA*⁻ *thyA*⁻ *endI*⁻ *dnaC*(Ts)] are temperature-sensitive mutants of H502 (*uvrA*⁻ *thyA*⁻ *endI*⁻) described previously (5, 6). *E. coli* strain C2309 [*uvrA*⁻ *thyA*(Ts) *dnaG*(Ts)] was obtained from R. Calendar.

The bacteriophage strains used in these experiments are listed in Table 1, as are the sources of the phage and some of their distinguishing properties.

Media and buffers. TPGA medium is TPG medium (15) with 1 g of KH₂PO₄ and 10 g of Casamino Acids per liter. SVB medium has been described (2). Buffer E consists of 40 mM Tris-acetate (pH 7.8), 5 mM sodium acetate, and 1 mM EDTA (16).

Isolation of phage RF DNA. Intracellular double-stranded RF DNA was isolated from chloramphenicol-treated, phage-infected *E. coli* cells by two different procedures. Host chromosomal DNA was removed from lysates of infected cells by the procedure of either Godson and Vapnek (9) or Hansen and Olsen (10). The phage RF DNA was further purified using phenol extraction, precipitation with ethanol, RNase digestion of contaminating RNA, and sedimentation through isokinetic sucrose gradients. Dialyzed DNA samples were stored in 50 mM Tris-hydrochloride-2 mM EDTA, pH 7.5, at -80°C.

Restriction endonuclease fragment analyses. The restriction endonucleases *Hae*III, *Hha*I, *Hinc*II, *Hpa*II, and *Pst*I were purchased from Bethesda Research Laboratories. Endonuclease *Eco*RI was purchased from Miles Laboratories. Reactions with DNA

TABLE 1. Sources and properties of bacteriophages

Phage	Property	Source
G4	Wild type	G. N. Godson
G4tr1	Temperature resistant	G. N. Godson
G4bs1	Temperature resistant	R. C. Warner
G4ev1	Temperature resistant	This laboratory
ϕ X174am3	Lysis defective	R. L. Sinsheimer
ϕ Kh-1	Host range mutant	A. Taketo

were carried out essentially as specified by these suppliers. The reactions (total volume, 50 μ l) were terminated after 1 h by the addition of 10 μ l of a solution containing 2 M sucrose, 100 mM EDTA (pH 8), and 0.04% (wt/vol) bromophenol blue.

The DNA fragments were separated by electrophoresis on 1% (wt/vol) agarose slab gels containing buffer E and 1 μ g of ethidium bromide per ml, as described by Sugden et al. (16). The running buffer (buffer E) included 0.5 μ g of ethidium bromide per ml. Electrophoresis was carried out at 100 V for approximately 2.5 h. The gels were photographed over a shortwave UV light box, using Polaroid type 47 film.

DNA synthesis rate measurements. Host bacteria were grown at 33°C on TPGA medium (80 ml) supplemented with 2 μ g of thymine per ml to cell densities of approximately 3×10^8 cells per ml. The cells were collected by centrifugation and suspended in 5 ml of SVB medium supplemented with 10 mM CaCl_2 . Phage were then added, G4 at a multiplicity of infection of 10 or ϕ X174am3 at a multiplicity of infection of 3. After 20 min of incubation at 33°C without aeration, 75 ml of TPGA medium supplemented with 1 μ g of thymine and 30 μ g of chloramphenicol per ml was added (zero time). Incubation was continued at 33°C for 120 min. At 20 and 40 min, portions of the culture were shifted to the restrictive temperature.

At 10-min intervals, 20 ml of each culture was transferred to separate vessels containing 10 μ Ci of [^3H]thymidine in 0.5 ml of TPGA medium and incubated for 2 min either at 33°C or at the restrictive temperature. Cold acetone was added to terminate the pulse. The cells were collected by centrifugation and suspended in 1 ml of 50 mM sodium tetraborate-10 mM EDTA, pH 9. Lysozyme (200 μ g) was added, and the mixtures were incubated for 15 min at room temperature. Potassium hydroxide was then added to a final concentration of 0.3 N, and the lysates were incubated overnight at 30°C. Calf thymus DNA carrier (200 μ g) and 2 ml of cold 10% trichloroacetic acid were added. The precipitates were collected on glass fiber filters. The amount of acid-insoluble radioactivity in each sample was then measured with a liquid scintillation spectrometer.

RESULTS

Two preliminary observations raised the possibility that G4tr1 and wild-type G4 were quite different phages. (See Table 1 for a description of these and the other phages used in these experiments.) DNA synthesis on G4tr1 single-stranded template DNA was defective in soluble protein extracts of the *E. coli dnaC* defective

mutant PC79, whereas DNA synthesis on wild-type G4 DNA was not (M. Bayne and L. Dumas, unpublished data). Second, G4bs1 formed plaques with high efficiency at 41°C on the *dnaB* defective mutant host strain LD311, whereas G4tr1 did not (D. Benesh and L. Dumas, unpublished data). Given these differences, we decided to determine whether G4tr1 is, in fact, a simple derivative of G4 or another bacteriophage.

Restriction endonuclease fragment analyses. Duplex RF DNA molecules were isolated from phage-infected cells. The analyses of the products of digestion with endonuclease *Pst*I showed that G4, G4tr1, G4bs1, and G4ev1 RF DNA molecules all carry a single cleavage site recognized by this enzyme. Endonuclease *Eco*RI also recognized a single cleavage site on G4, G4bs1, and G4ev1 RF DNA, but no site on G4tr1 DNA. These data (not shown) suggest that G4tr1 RF DNA is different from that of G4, G4bs1, and G4ev1.

The uniqueness of G4tr1 DNA is even more apparent from the data shown in Fig. 1. Digestion of G4, G4bs1, and G4ev1 RF DNA with endonuclease *Hpa*II yielded DNA fragments with identical sizes upon separation by electrophoresis in agarose gels (Fig. 1a). The number and sizes of fragments generated from G4tr1 DNA were different from those of the other G4 phages and from ϕ Kh-1 and ϕ X174. Similarly, Fig. 1b shows that digestion of G4, G4bs1, and G4ev1 RF DNA molecules with endonuclease *Hinc*II yielded identical products. The fragment pattern of G4tr1 DNA again differed from the other G4 phages, from ϕ Kh-1, and from ϕ X174. It is clear, therefore, that G4tr1 is unlike these other phages. Agarose gel electrophoresis of endonuclease *Hae*III and *Hha*I digests yielded data consistent with this conclusion.

Host proteins required for G4 RF DNA replication. Since G4tr1 is not a simple derivative of G4, previously published data using this phage (3) cannot be taken as indicative of host cell DNA synthesis protein requirements for G4 RF DNA replication. In addition, the data obtained using wild-type G4 (12) are not sufficient for establishing these host requirements because of the inherent temperature sensitivity of the phage. We found that the inhibition of G4 RF DNA replication observed, for example, in the *dnaC* mutant host at the restrictive temperature (12) was also evident in temperature-insensitive revertants of the mutant host (unpublished data). Thus, data from these experiments cannot be used to argue that the host cell *dnaC* protein is required for G4 RF DNA replication. Rather, experiments of this kind must be carried out with G4 temperature-resistant phages. We have

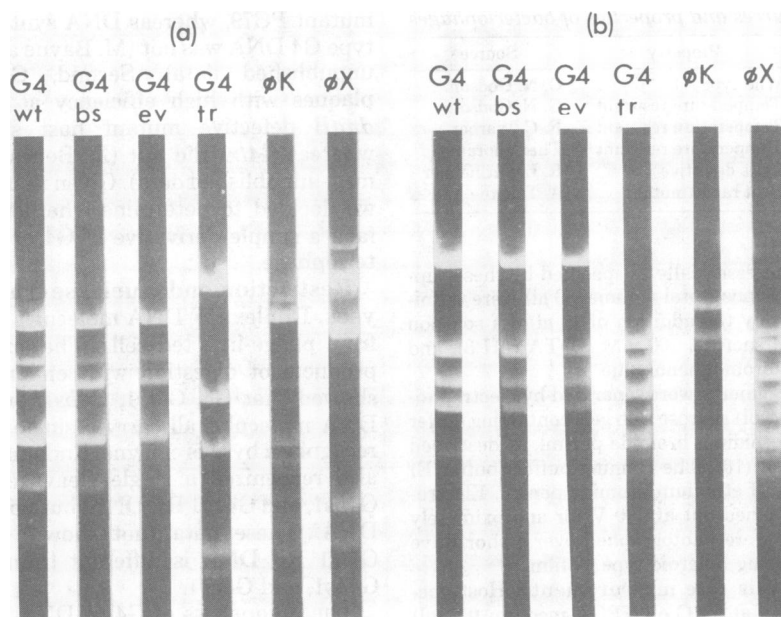


FIG. 1. Agarose gel electrophoresis of the restriction endonuclease digestion products of RF DNA. Phage G4, G4bs1, G4ev1, G4tr1, ϕ Kh-1, and ϕ X174am3 RF DNA molecules were cleaved with HpaII (a) and HincII (b). The digestion products were separated by electrophoresis as described in the text.

used G4ev1 and G4bs1 in a series of such experiments.

We infected mutant and revertant host cell cultures containing 30 μ g of chloramphenicol per ml with G4ev1, shifting portions of the cultures to the restrictive temperature at 20 and 40 min after infection. The cells had been treated with mitomycin C before infection to selectively inhibit host DNA synthesis. The rates of G4 DNA replication were measured at 10-min intervals. Essentially all of the radioactive DNA made under the conditions of these pulse-label experiments in all host strains used was found in duplex RF DNA (data not shown).

Figure 2 shows the results of measurements of rates of G4ev1 RF DNA replication in the *dnaB* mutant host LD311. Shifts from the permissive temperature to the restrictive temperature resulted in similar changes in rate of RF DNA replication in the mutant host (Fig. 2A) and the temperature-insensitive revertant host (Fig. 2B). The rate of ϕ X174 RF DNA replication was markedly reduced upon shifting to 40°C at 20 and 40 min after infection (Fig. 2C), indicating that the *dnaB* protein was defective at the elevated temperature. The observation that G4ev1 RF DNA replication was not inhibited under conditions in which the *dnaB* protein was defective indicates that this protein is not essential for replication of the duplex G4 DNA molecule. The results of analogous experiments using

G4bs1 (data not shown) were consistent with this conclusion.

In similar experiments, G4ev1 RF DNA replication rates were measured at permissive and restrictive temperatures in the *dnaC* defective mutant host LD332 and its temperature-insensitive revertant. The results of these experiments are shown in Fig. 3. Again, shifts to the elevated temperature did not result in more reduction of the rate of RF DNA replication in the mutant host than in the revertant (compare Fig. 3A to B). The fact that the *dnaC* protein was defective under these conditions was documented by the observation that ϕ X174 RF DNA replication was extremely slow, even at 33°C (Fig. 3C). Previous experiments (6, 13) have shown that the *dnaC* protein activity is diminished in this host even at the permissive temperature. The amounts of labeled DNA synthesized in the ϕ X174-infected culture were near the background levels seen in uninfected cell cultures under these conditions and more than 20-fold lower than the amounts synthesized in ϕ X174-infected revertant cultures (data not shown).

Since G4 RF DNA replication was no more temperature sensitive in the mutant host than in the revertant, the *dnaC* protein does not appear to be essential. The results of similar experiments with G4bs1 (data not shown) were consistent with this conclusion.

The host cell *dnaG* protein is essential for G4

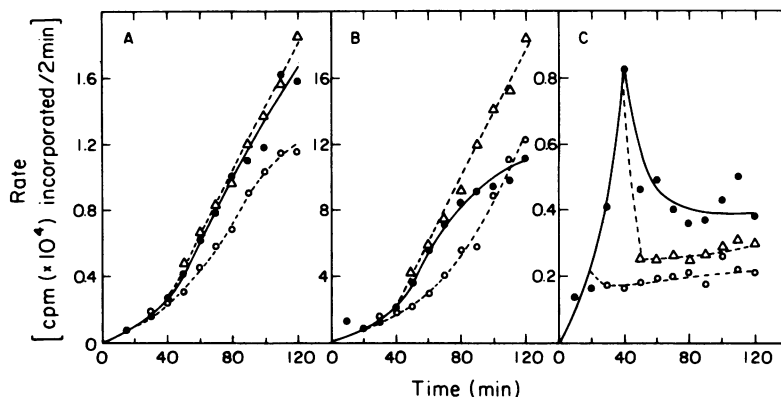


FIG. 2. RF DNA replication rates in the *dnaB* mutant host strain LD311. (A) *G4ev1*-infected LD311; (B) *G4ev1*-infected LD311R, a spontaneous temperature-resistant revertant of LD311; (C) ϕ X174-infected LD311. Symbols: ●, rates at 33°C; ○, rates at 40°C, shifted from 33 to 40°C at 20 min; △, rates at 40°C, shifted at 40 min.

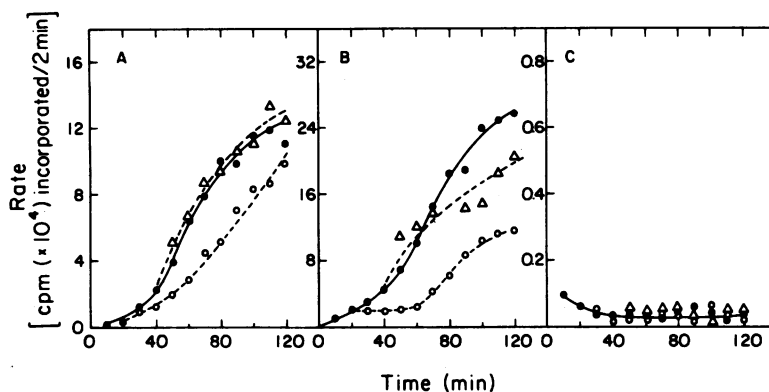


FIG. 3. RF DNA replication rates in the *dnaC* mutant host strain LD332. (A) *G4ev1*-infected LD332; (B) *G4ev1*-infected LD332R, a spontaneous temperature-resistant revertant of LD332; (C) ϕ X174-infected LD332. The symbols are the same as in Fig. 2.

RF DNA replication. Figure 4 shows that *G4ev1* RF DNA replication rates were more sensitive to shifts to the restrictive temperature in the mutant host (Fig. 4A) than in the revertant (Fig. 4B). This control demonstrates that the requirements for essential host cell proteins can be clearly demonstrated with these measurements.

DISCUSSION

Bacteriophages *G4ev1* and *G4bs1* have been designated as derivatives of wild-type G4 on the basis of the identical fragment patterns obtained upon digestion with endonucleases *EcoRI*, *PstI*, *HincII*, *HpaIII*, *HaeIII*, and *HhaI*. *G4ev1* and *G4bs1* are less inherently temperature sensitive than wild-type G4. Measurements of the rates of *G4ev1* RF DNA replication at the restrictive temperature in *dnaB* and *dnaC* mutants of *E. coli* and their revertants show that RF DNA

replication continues at normal rates. This indicates that the protein products of these two genes are not essential for normal *G4ev1* RF DNA replication. In contrast, RF DNA replication is inhibited at the restrictive temperature in a *dnaG* mutant host. The product of this host gene is required for normal RF DNA replication. These observations are consistent with those made using *G4bs1*-infected cells.

Our conclusion that *G4ev1* and *G4bs1* phages do not require the host cell *dnaB* and *dnaC* proteins for normal RF DNA replication differs from the interpretations of the data from previous experiments with *G4tr1* (3) and G4 wild-type (12). The restriction endonuclease digest data presented here demonstrate that *G4tr1* is different from G4. Therefore, the fact that *G4tr1* requires the host cell *dnaB* and *dnaC* proteins for RF DNA replication is not inconsistent with the observation that G4 does not. Rather, it

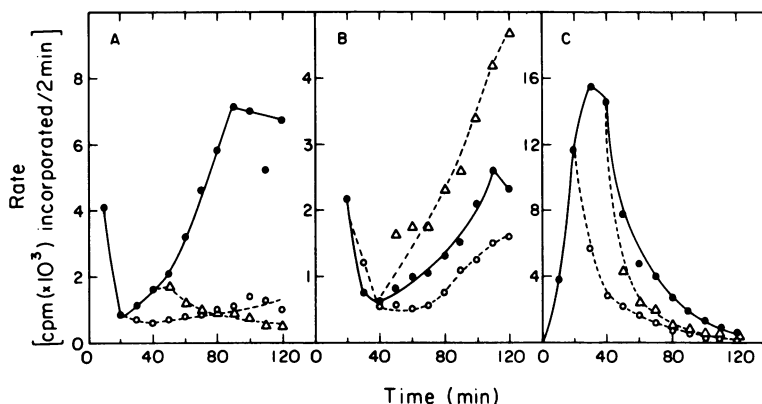


FIG. 4. RF DNA replication rates in the *dnaG* mutant host strain C2309. (A) G4ev1-infected C2309; (B) G4ev1-infected C2309R, a spontaneous temperature-resistant revertant of C2309; (C) ϕ X174-infected C2309. The symbols are the same as in Fig. 2.

supports our contention that G4tr1 is a misnomer for a phage that is quite different from G4.

Also, our data from wild-type G4-infected host mutants (data not shown) do not differ from those published previously (12), but our interpretations do. We do not interpret the temperature sensitivity of RF DNA replication in *dnaB* and *dnaC* mutant hosts to be due to the *dnaB* and *dnaC* mutations, respectively, since we observed similar temperature sensitivity in temperature-insensitive revertants of these host mutants. Instead, we argue that valid interpretations require the use of temperature-resistant strains of G4 and data from infections of revertant hosts as well as mutant hosts.

Thus, the requirements for G4 RF DNA replication are similar to those of other group II isometric phages such as ϕ K, St-1, and α -3 (for review, see reference 4), in that the *dnaG* protein, but not the *dnaB* and *dnaC* proteins, is required for DNA chain initiation. Group I isometric phages, such as ϕ X174, S13, and ϕ A (4), however, do require the *dnaB* and *dnaC* proteins to assist the *dnaG* protein in this process.

The *dnaG* protein is able to catalyze the synthesis of oligonucleotide primers on the free viral strands of G4 DNA in vitro and in vivo without the assistance of the *dnaB* and *dnaC* proteins (1, 11, 14, 17). Thus, the host cell DNA initiation protein requirements for circular G4 RF DNA replication are consistent with the replication model proposed by Fiddes et al. (7) and with the simplest version of the rolling-circle model (8). According to these models, cleavage of the viral DNA strand of the duplex RF molecule at the origin of replication provides a 3'-OH primer terminus for chain elongation, using the complementary strand as template. No de novo chain initiation is required. Displacement of the viral

template strand from its 5' end eventually exposes the same de novo chain initiation site recognized by the *dnaG* protein during the conversion of the free infecting viral strand to its duplex replicative form early in infection. De novo chain initiation catalyzed by the *dnaG* protein occurs at this same site during RF replication without the need for the *dnaB* and *dnaC* proteins. G4 escapes the need for these latter two initiation proteins, as do other group II isometric phages, simply because the viral strand carries a site recognized by the *dnaG* protein. A single de novo chain initiation site on the viral strand, therefore, is sufficient for the RF DNA replication process.

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